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Hakija
Applicant

1. KORPELA, Matti
   Naantali
2. KARP, Matti
   Kaarina
3. KURITTU, Jussi
   Turku

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A NEW ASSAY METHOD

FIELD OF THE INVENTION

This invention relates to a method for the determination of a tetracycline in a sample. The invention also concerns recombinant prokaryotic cells capable of emitting light in response to the existence of a tetracycline in a sample. Furthermore, the invention relates to novel DNA vectors useful for the construction of said prokaryotic cells.

BACKGROUND OF THE INVENTION

The publications and other materials used herein to illuminate the background of the invention, and in particular, cases to provide additional details respecting the practice, are incorporated by reference.

Whole-cells can be used in methods based on the use of living cells or organisms as sensor tools of detection. Many of these methods utilize bacterial or yeast cells. Prokaryotic organisms and especially *Escherichia coli* bacterium are very well characterized and maps of genes and their sequences at nucleotide level are known. Therefore the behaviour of the whole cell sensor can be better understood. Because of this fact it is also possible to develop analyte or group specific sensors utilizing different regulatory regions of genomes and also various microbial strains. Seven complete genomes are currently available in public databases (*Haemophilus influenzae* (Fleischmann *et al.*, 1995, Science, 269, 496-512), *Mycoplasma genitalium* (Fraser *et al.*, 1995, Science, 270, 397-403), *Methanococcus jannaschii* (Bult *et al.*, 1996, Science, 273, 1058-1073), *Synechocystis* sp. PCC6803 (Kaneko, T. et al. (1996). Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome .

Res. 3, 109-136), *Mycoplasma pneumoniae* (R. Himmelreich et al., (1996) Complete sequence analysis of the genome of the bacterium Mycoplasma pneumoniae. Nucleic. Acids Res. 24(22): 4420-4449), *Saccharomyces cerevisiae* (A. Goffeau et al (1997) The Yeast Genome Directory. Nature Supplement, Vol. 387 No.6632) and *Escherichia coli* (Blattner FR, Plunkett G 3rd, Bloch CA, Perna NT, Burland V, Riley M, Collado-Vides J, Glasner JD, Rode CK, Mayhew GF, Gregor J, Davis NW, Kirkpatrick HA, Goeden MA, Rose DJ, Mau B, Shao Y (1997) The complete genome sequence of *Escherichia coli* K-12. Science 277:1453-1474). Based on information reported at the Small Genomes Conference, held in Hilton Head, South Carolina, in January 1997, as many as 30 projects for sequencing complete genomes are planned or underway. Some of these are listed on the World Wide Web site at http://www.tigr.org/tdb/mdb/mdb.html. The sequencing project of whole-genome structure of *Bacillus subtilis* has recently resulted in the knowledge of the genomic organization at nucleotide level. The data however, has not yet been published.

Whole cells can be utilized in biosensors which are devices consisting of 1) a sensor, 2) a recording unit and 3) a possible connector such as fiber optic guide between 1 and 2. In such biosensors the sensor cells should be situated in the sensor in immobilized form. Immobilization of the sensor cells can be done by passive or active means such as entrapping the cells in alginate, agarose or polyacrylamide gel or by covalently binding them in a matrix. Also immobilization of the cells as freely swimming in a nutrient solution behind a semipermeable membrane comes into guestion. Essential in this kind of a biosensor is that the sensor cells are in close contact with the analyte to be assayed and in the intimate contact with the recording unit. The recording unit has several choices on what is the physical background of the measurement. It can be change in heat, conductance, colour reaction, chaag

fluorescent properties, emission of endogenous light from the sensor cells etc.

Conventional tests for the measurement of toxic substances such as antimicrobial agents (antibiotics) are based on the inhibition of growth. The growth inhibition can be followed by simple growth inhibition zone of microbes on a nutrient agar plate around a disk onto which an antibiotic dilution was pipetted. Typical examples are cylindrical, hole or disk methods to make agar diffusion tests. The difference in these tests is only restricted in the way how the sample is applied on the agar and also on the way of usage of the bacteria in the test. Another means is to follow the metabolism of the test organisms by estimating the intensity of a color reaction which is affected by the inhibitory antibiotic present and comparing it to the uninhibited control (e.g. the commercial product Delvo Test™, Gist-Brocades, Brilliant black-reduction test, Charm Farm Test, Charm AIM-96 and Valio T101-test). Since microbiological methods utilize bacteria or their spores it is the sensitivity of the test bacteria which is of utmost importance. Thus far one had to make compromises in the choice of suitable test strain since great sensitivity against antimicrobial agents and other characteristics needed for the test strain have not been common features for the same strain of bacteria. A major drawback when using microbes in antibiotic residues tests is slow and unsensitive performance. Since in these methods one always controls in a way or other the growth of tester strain one cannot imagine the test to be performed in an hour. This is due to the fact that growth of microbe is a slow phenomenon even at it's fastest mode. Also in many cases microbes are in spores or freeze-dried, the regeneration of which makes the tests even more slow to perform.

There has been a big need to develop faster tests for residue testing. These tests normally use

or monoclonals). In 1979 the first rapid β-lactam test The Charm I test was introduced. This test used radioactive tracers binding to microbial receptors. In 1984 the Charm II test was launched and this test used binding sites of various bacteria to allow broad antibiotic residue spectrum (e.g. β-lactam, tetracyclines, aminoglycosides, chloramphenicol, sulfa drugs and macrolides). Penzyme test is based on inhibition of receptor connected transpeptidase by β-lactam antibiotic. There are several tests using either purified receptors or antibodies for antibiotic residue testing: IDEXX SNAP test (enzymatic test), Delvo-X-PRESS (immunological test), Penzym 100 (enzymatic test), Penzym S100 (enzymatic test), LacTek Milk Screening Kit (immunological test) and Idetek LacTek BL (immunological test).

Antibiotic detection methods based on bioluminescence measurement have been developed. Ulizur (1986, Methods Enzymol., 133, 275-284) described three different ways to use bioluminescence for the detection of antimicrobial agents: a) lysis test, b) induction test and c) bacteriophage test.

In the lysis test the *lux*-genes isolated from *Vibrio fischeri* produce luciferase protein which in the presence of substrates produces light. The genes have been coupled into a plasmid, which has been transferred to *Bacillus subtilis*, which is sensitive to antibiotics affecting bacterial membranes such as penicillins and kefalosporins. In the test the *B. subtilis* containing the *lux*-genes is grown together with a sample. If there is antibiotic present the synthesis of cell wall components is prevented and bacteria are lysed, thus yielding a lower light emission level compared to blank.

The induction test is based on the use of dim mutants of

light. This as also other bioluminescence tests developed by Ulizur are based on chromosomal DNA of the target cell. The induction test enables the detection of antibiotics affecting protein synthesis. When the bacteria are incubated together with compounds binding to DNA they start to produce light, i.e. protein synthesis is initiated. If there is present any antibiotic affecting on protein synthesis there is a decline in light emission obtained. Again the amount of antibiotic present is quantitated when compared to blank without antibiotic. This method does not enable the detection of antibiotics affecting DNA synthesis. Also the actual performance of the method is questionable because the basis of the method is not well known. To perform the test it is essential to add minimal salts (such as Ca^{2+} and Mg^{2+} -ions) which are known to diminish or completely prevent the action of aminoglycosides (tetracycline, streptomycin, kanamycin, neomycin, erythromycin) action. Also the induction parameters are very strict and if the samples contain other antibiotics (for instance nalidixic acid) or other ~~substances triggering light production there might exist~~ problems in the evaluation of results. Thus, in this test the difficulty might be the great number of inducers. Also the amount of bacteria in the test have been claimed to be a critical parameter. If there exists too high concentration of bacteria, the test has to be aerated due to absolute requirement of oxygen for the bioluminescence reaction in these bacteria. This results again in problems ~~when special measuring devices are used, and also the~~ repetitity of the assay is affected due to these facts.

The bacteriophage test enables the detection antibiotics affecting DNA-synthesis, transcription and translation. In this test wild-type, light-emitting *P. phosphoreum* bacteria are infected with lytic bacteriophages. In the presence of antibiotic there cannot be synthesized new infective phages [illegible] synthesis is

is unchanged compared to initial light level. However, if there does not exist any antibiotic, the phages rapidly multiply and inactivate host bacteria thus making it incapable of producing light. The bacteriophage test is difficult to perform since it is necessary to add phages (sometimes with different titers) to the assay mixture and the timing of the addition of antibiotic has to be very careful. Also this test suffer from the same problems as those in the induction test with respect to the composition of assay mixture and the amount of bacteria used in the assay. It is not possible to detect single antimicrobial agents or groups of them with the presently used microbial methods for antibiotics. Instead, the methods reveal all antibiotics against which the test microbe is sensitive. By changing measuring conditions or by adding enzymes degrading certain compounds it is possible to block the effect of some antibiotics.

Antibiotics used as medicins against microbial invasion are detected from body fluids in order to study the dosage and penetration of the medicine. Often the effective therapeutic range of the antibiotic is rather narrow and the risks from overdosage might be too big. It is also important to measure the presence/concentration of antibiotics from meat and milk due to syndrome for allergic people. In the course of cheese production there should not exist antibiotics in milk used as starting material due to the fact that cheesemaking bacteria are not able to work on contaminated milk. The common methods to detect antimicrobial medicines are microbial methods performed on the lawn of agar. The direct method is based on the measurement of the inhibition of the growth of sensitive bacterial strains. Alternatively, one can measure some metabolic parameter such as acid production of a sensitive strain of bacteria using different colour reactions.

Recombinant DNA plasmids, where the production of a for...

regulatorable promoter, has been developed during last twenty years at exponential speed. In many cases the goal has been to create as high production of foreign protein as possible in the new organism such as in *E. coli*. In these cases the production of foreign protein can yield as much as 25 % of total cellular protein (Caulcott & Rhodes, 1986, Trends in Biotech., June, 142-146). If so high amounts of protein are produced it is obvious, that this production is deletorious to host cell and its metabolism. Due to the harmful effects, several plasmids where the production of foreign protein has been put under the control of a regulatable promoter, have been developed. The production of protein can here be turned on at optimal growth phase of the microbe. In these cases the cultivation of the microbe is performed in unstressing conditions until growth has reached cell density suitable for maximal production of the protein. After this, protein production is switched on by adding a chemical to the medium which triggers the production. Also change in physical parameters such as increase or decrease in cultivation temperature in certain ~~cases cause the protein production.~~

The plasmids commonly used contain one or more resistance factors useful for the selection from large population of cells only those who contain the plasmid. The resistance factor helps cell to survive in circumstances, which are poisonous to other cells. The selecting factor is added in the growth medium to prevent other cells growth except for ~~the one containing the plasmid. The resistance determinant~~ is a gene which codes for protein which degrades or otherwise inactivates the poisoneous factor (which can be for instance an antibiotic) present in the growth medium. Several genes encoding resistance factors are known. The most frequently used gene is the gene coding for β-lactamase which is able to degrade penicillins, or β-lactams which are their derivatives. As the result, the [illegible] of penicillin is lost and bacteria can

for chloramphenicol acetyltransferase, kanamycin acetyltransferase and tetrahydrofolate reductase. Depending on the type of cells used also genes which carry ability for the cell to grow in the presence of tetracycline, erythoromycin, spectinomycin, streptomycin, sulfonamides, neomycin, thiostrepton, viomycin and colisins. It is also known resistance factors which eliminate or change the heavy metal present in the medium. Selection pressure in favor of cells containing a plasmid can also be achieved by transferring a gene encoding a function which complements a growth defect, which lacks from the chromosome of the organism. These kind of genes are normally those which code for factors participating in amino acid biosynthesis pathways. In these cases certain vital amino acid is in short from the growth medium and cell can not grow unless the gene present in the plasmid produces the enzyme synhesising amino acid in question or it's intermediate. Also other vital functions result in a beneficial situation for those cells containing the plasmid compared to cells without the plasmid. The plasmid can contain for instance a ~~gene encoding protein which participates in the formation~~ of cell wall components or heritable material.

OBJECT AND SUMMARY OF THE INVENTION

The object of the invention is to provide a novel method of determining a tertracycline in a sample where said method is rapid and selective for tetracyclines, i.e. the method ~~is able to distinguish tetracyclines from other~~ antimicrobial agents.

According to one aspect of the invention, there is provided a method for the detemination of a tetracycline in a sample, wherein the method is characterized in that
- the sample is brought into contact with cells encompassing a DNA vector including a nucleotide sequence encoding a light producing enzyme under transcriptional

promoter,
- detecting the luminescense emitted from the cells, and
- comparing the emitted luminescence to the luminescence emitted from cells in a control containing no tetracycline.

According to another aspect, the invention concerns a recombinant prokaryotic cell which encompasses a DNA vector including a nucleotide sequence encoding a light producing enzyme, tetracycline repressor and tetracycline promoter.

According to yet another aspect, the invention concerns a plasmid which comprises either
- the luxCDABE genes, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10, or
- the insect luciferase gene, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10.

BRIEF DESCRIPTION OF THE DRAWINGS

~~Figure 1a shows schematically the method according this~~ invention, where cells cloned with the plasmid pTetLux1 are used,

Figure 1b shows schematically the method according this invention, where cells cloned with the plasmid pTetLuc1 are used,

~~Figure 1c shows the schematically the production of the~~ luciferase enzyme,

Figure 2a shows the plasmid pTetLux1,

Figures 2b to 2e show the nucleotide sequence of the plasmid pTetLux1,

~~the plasmid pTetLuc1~~

Figures 3b to 3f show the nucleotide sequence of the plasmid pTetLuc1,

Figure 4a shows the production of light (induction coefficient) versus concentration of tetracycline in samples for three different tetracyclines,

Figure 4b shows the production of light (induction coefficient) versus concentration of tetracycline in samples for further four different tetracyclines,

Figure 5 shows the effect of magnesium ions on the sensivity of the method according to the invention,

Figure 6 illustates possibilities of changing the assay window for the method of the invention by adjusting magnesium ion concentration and pH.

Figure 7 shows the assay using freeze-dried *E. coli* in the determination of tetracycline,

Figure 8 shows a comparison of the assays based using cells with the plasmid pTetLuc1 and with the plasmid pTetLux1,

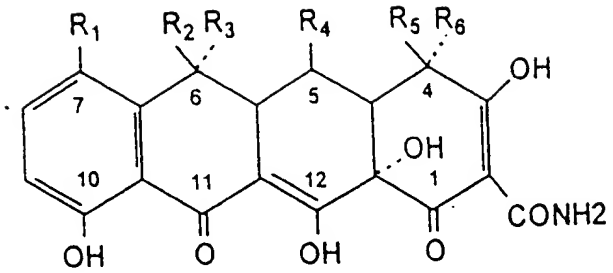
Figure 9 shows an assay of a pig serum sample (cells *E. coli* K12, pTetLux1),

Figure 10 shows the effect of EDTA in a milk sample assay, and

Figure 11 shows the light emission versus time for an assay according to the invention.

DETAILED DESCRIPTION OF THE INVENTION

The term "tetracycline" shall be understood to include any compound covered by the general



and particularly the specific commerially available compounds listed in the table below.

GENERIC NAME	R_1	R_2	R_3	R_4	R_5	R_6
Chlorotetracycline	Cl	OH	CH_3	H	H	$N(CH_3)_2$
Demethylchlorotetracycline	Cl	OH	H	H	H	$N(CH_3)_2$
Doxycycline	H	H	CH_3	OH	H	$N(CH_3)_2$
Methacycline	H	CH2	H	OH	H	$N(CH_3)_2$
Minocycline	$N(CH_3)_2$	H	H	H	H	$N(CH_3)_2$
Oxytetracycline	H	OH	CH_3	OH	H	$N(CH_3)_2$
Tetracycline	H	OH	CH_3	H	H	$N(CH_3)_2$

Furthermore, the term "tetracycline" shall be understood to cover the metabolic and other reformulation/decomposition products thereof.

The cells useful in the method of the invention are preferably prokaryotic cells. Especially preferable are the bacteria *Escherichia coli*, which are stored in dried form, in the method

can be used.

According to a preferred embodiment, the DNA vector including a nucleotide sequence encoding a light producing enzyme is a plasmid containing the luxCDABE genes, tetracycline repressor (TetR) and tetracycline promotor (TetA) from transposon *Tn*10. Particularly preferable is the plasmid pTetLux1.

According to another preferred embodiment, the DNA vector including a nucleotide sequence encoding a light producing enzyme is a plasmid containing the insect luciferase gene, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10. In this case the substrate for insect luciferase reaction, D-luciferin, is added to the mixture of the sample and the cells in order to initiate the luminescence of the cells. The plasmid is preferably pTetLuc1.

The method according to this invention is useful for the determination of tetracycline in various kinds of samples. As examples can be mentioned milk, fish, meat, infant formula, eggs, honey, vegetables, serum, plasma, whole blood or the like.

The luminescence of the cells is preferably measured using an X-ray or polaroid film, a CCD-camera (Charge Coupled Device), a liquid scintillation counter or, most preferably, a luminometer.

The sensitivity of this analysis method with respect to the tetracycline can be controlled by increasing or decreasing the concentration of divalent metal ions, e.g. magnesium ions, in the mixture of the sample and the cells, by adjusting the pH or by combinated adjusting of the divalent metal ion concentration and the pH. Increasing concentration of magnesium ions decreases the sensitivity

sensitivity. The sensitivity of the analysis with respect to the tetracycline can be increased by the use of cells which are especially antibiotic sensitive mutant strains. Chelating agents such as EDTA can be added to further sensitize the sensor system for tetracyclines.

Figures 1 show a schematic representation of a method based on specific detection of the presence of tetracyclines using microbial cells cloned with either the plasmid pTetLux1 (Figure 1a) or with the plasmid pTetLuc1 (Figure 1b). The figures show that cells containing either of the plasmids can be triggered to produce light by adding a chemical agent (a tetracycline). The light production is a consequence of tetracycline responsive promoter activation due to removal of the tet-repressor protein leading to the production of luciferase specific mRNA and luciferase protein itself. The principle is demonstrated in Figure 1c. In case of the usage of full length bacterial luciferase operon containing *lux*C, *lux*D, *lux*A, *lux*B and *lux*E genes (Figure 1a) one is able to get light emission without addition of any substance. In case of insect (e.g. firefly) luciferase (Figure 1b) light is emitted only after the addition of D-luciferin. It should be noticed that the triggering of luciferase synthesis and light production commences immediately the cells are introduced for the inducer molecules (tetracyclines). Therefore there is no need to use dividing cells and hence there is no need to use long cultivation of microbial cells such as the case is with conventional methods. Therefore, if needed, one can get results in minutes rather than in hours or days which is the case when conventional methods are used.

Figure 2a shows the plasmid pTetLux1, in which the production of bacterial luciferase of *Photorhabdus luminescens* (formerly *Xenorhabdus luminescens*; the lux-operon structure and the full-length nucleotide sequence of

(1990) Nucleotide sequence, expression, and properties of luciferase coded by lux genes from a terrestrial bacterium. J. Biol. Chem. 265:16581-16587) can be switched on by the addition of chemical agent belonging to the tetracycline family of antimicrobial agents in a cloned *E. coli* bacterium. Figure 2b shows the nucleotide sequence of the plasmid pTetLux1. This plasmid construct is devised to contain the five genes from *P. luminescens* luciferase operon necessary for the light production without any additions of substrates, i.e. cells cloned with such a construct produce substrates endogenously. By incubating *E. coli* cells containing this plasmid (or any other microbial strain whereto similar regulation/reporter gene system is incorporated containing the necessary secondary regulatory sequences in the constructs such as correct ribosome binding region, transcriptional termination etc) in the presence of very small amounts of tetracyclines one is able to obtain light production the intensity of which is proportional to the concentration of tetracycline used.

Any *E. coli* mutant strain and especially those strains having a mutation in the export/import machinery of the membranes or otherwise leaky character making it possible for large molecules to easily penetrate inside the cell would be benefial to use in the method described in this invention. Also other gram-negative bacteria such as strains belonging to genus Salmonella, Shigella, Enterobacter, Citrobacter, Klebsiella, Erwinia, Pseudomonas, Serratia as well as gram-positive organisms such as those belonging to genus Bacillus (especially B. subtilis, B. licheniformis, B. pumilus, B. globigii, B. natto, B. amyloliquefaciens and B. niger, B. brevis, megaterium), Streptomyces, Lactobacillus (especially L. lactis, L. casei) and Streptococcus (especially S. thermophilus, S. cremoris, S. agalactiae) come into question. Especially asporogenic strains of Bacilli or Lactobacilli are suitable

Figure 3a shows the plasmid pTetLuc1, in which the production of firefly luciferase of *Photinus pyralis* (The gene encoding firefly luciferase was originally cloned and seuenced in the middle of the 1980's by DeWet, J. et al. (1987) Firefly luciferase gene: Structure and expression in mammalian cells, Mol. Cell. Biol., 7, 725-737) can be switched on by the addition of chemical agent belonging to the tetracycline family of antimicrobial agents in a cloned *E. coli* bacterium. Figures 3b to 3f show the nucleotide sequence of this plasmid. By incubating *E. coli* cells containing this plasmid (or any other microbial strain whereto similar regulation/reporter gene system is incorporated containing the necessary secondary regulatory sequences in the constructs such as correct ribosome binding region, transcriptional termination etc) in the presence of very small amounts of tetracyclines one is able to obtain light production by the addition of D-luciferin, which is the substrate of firefly luciferase. The intensity of light emission is proportional to the concentration of tetracycline used.

Figures 4a and 4b shows the effect of altogether seven different tetracyclines on the production of light as a function of concentration of each tetracycline. As controls different non-tetracycline antibiotics were included in this study to show that the sensor strain is specific for the tetracyclines. The luminescense was emitted from *E. coli* containing the plasmid pTetLux1. The detection was made after an incubation of 90 min. All tetracyclines tested behaved in a very similar manner and induction efficiences were at the same antibiotic concentration area. This makes this sensor even more attractive for analytical use of assay of tetracycline group of antibiotics.

It should be noted that the accumulation of various tetracyclines into microbial cells is very strongly dependent of Mg^{2+}-ions.

There is shown in Figure 5 the effect of increasing concentrations of Mg^{2+}-ions on the behavior of *E. coli* cells containing the plasmid pTetLux1. As seen the tetracycline response curve is shifted to the right as a function of increasing concentrations of added Mg^{2+}- ions. Thus by increasing the Mg^{2+}-ion concentration one is able to decrease the sensitivity of the tetracycline sensor described in this invention. This fact is of great importance in cases where one does not need a high sensitivity of the measurement and where the approximately concentration of the ion is roughly constant and known such as in milk, serum and plasma.

The sensivity can be increased by removing magnesium ions from the assay mixture e.g. by adding a chelating agent forming a complex with magnesium.

Figure 6 shows the possibilities to change the assay window for tetracyclines by adjusting the magnesium ion concentration and by combined adjustment of the magnesium ion concentration and the pH.

The sensitivity of the assay can be increased by the use of cells which are especially antibiotic sensitive mutant strains. Hundreds of specific mutations for bacteria are known with which it is possible to study the activity of specific reactions. For instance trace amounts of antibiotics cause visible changes in the metabolism or in the cell membranes of antibiotic sensitive bacterial mutants. Mutations in cell wall structural components or biosynthetic enzymes as well as in transport and efflux proteins such as porins might have an effect on the behaviour of each sensor. Using these kind of mutations one is able to develop tests measuring residual antibiotics from biological material very sensitively. It is also rather simple to transfer new characteristics into bacterial cells by genetic engi[illegible]

phenomenon broadens the applicability of these organisms in tests utilizing whole cell sensor.

The measurement of the light emission can be done e.g. by using X-ray or polaroid film, using liquid scintillation counter or a luminometer. A special case in the use of CCD-camera (CCD = Charge Coupled Device) which can measure light emission on surfaces either directly or via a connector through a microscope. An important benefit in luminescent methods is the possibility to calibrate internally the measurements by using inside the same cell other genes which encode luciferase emitting different color which could be measured with special two wavelength-detecting apparatus. The other gene can be cloned in the same rec-DNA vector, in an other vector belonging to a different incombatibility group, inserted in the host chromosome, it can be carried in a phage etc. An example is the click beetle luciferases, which emit four different colors the wavelenghts ranging from 547 nm to 593 nm (Wood *et al.*, 1989, Science, 244, 700-702). The other gene resulting in different wavelenght can be put under inducible production system (indicator "gene") or it can be expressed constitutively (internal standard) to compensate possible secondary effects arising from heterologous samples.

Measurement of the light emission can be done by using X-ray or polaroid film, using liquid scintillation counter, a CCD-camera or a luminometer. The CCD-camera is an instrument which is capable of detecting very low levels of light. It is a compilation of light sensitive microchips which can record light emission from a microscopic or macroscopic target. In the applications of this invention such kind of a device could be used for the detection of tetracycline residues in food material such as vegetables or meat. The detection of light emission could be directly

chemiluminescent (such as peroxidase - luminol) or bioluminescent (such as luciferase - luciferin) reactions can be utilized. The luminometric method is performed with the aid of genes encoding either bacterial or beetle luciferases such as those described in the Figure 2 and 4. There exist several luminescent bacterial species such as *V. harveyi*, *V. fischeri*, *P. leiognathi*, *P.phosphoreum*, *Xenorhabdus luminescens* etc. Luminescent beetles are for example *Luciola mingrelica*, *Photinus pyralis*, *Pyrophorus plagiophthalamus*, *Lampyris noctiluca*, *Pholas dactylus*, etc. There exist also several eucaryotic species in the sea which luminesce, such as marine ostracod *Vargula hilgendorfii*, jellyfish *Aequorea victoria*, batrachoidid fish *Porichtys notatus*, pempherid fish *Parapriacanthus ransonneti* etc. Fluorescent reporter proteins such as green fluorescent protein (GFP) or any of it's variants could be used in the methods described in this invention (Li, X. et al. , 1997, Deletions of the aequorea victoria green fluorescent protein define the minimal domain required for fluorescence. J. Biol. Chem. 272(45), 28545-28549). In this invention high detection sensitivity of the luminescent enzyme labels inside a living cell associated with tetracycline-specific induction of label synthesis is based on the use of optimal concentration of all the reactants inside the cell including the necessary cofactors and acessory enzymes. All luciferase genes from these organisms would presumably work in a similar manner as the two examples shown in this invention. These systems together with enhancers and modulators (wavelenght, emission kinetics etc.) of light emission has been described in more detail in Campbell, A. "Chemiluminescence; principles and applications in biology and medicine", Weinheim; Deerfield Beach, Fl.; VCH; Chichester: Horwood, 1988.

Gossen and Bujard described a tetracycline-controlled expression of recombinant proteins in eucaryotic cells

in eucaryotic cells by tetracycline-responsive promoteres. US patent 5,464,758). Their system is based on a a procaryotic tet repressor and a protein capable of activating transcription in eucaryotic cells. Their invention relates to the use of a polynucleotide molecule in a method to regulate the expression of a heterologous gene sequence. This is operably linked to a promoter comprising, for example, a part of the cytomegalovirus promoter IE and at least one tet-operator sequence. As a model gene inserted under the control of tetracycline resposive elements they used firefly luciferase which is often used as a sensitive reporter gene in molecular biology research. The main differences to the invention described here are:

- The expression system is developed to express foreign proteins in eucaryotic cells for the purposes of production only under tight control of tet-regulatory system. The eucaryotic cells were described to be cell lines and strains which can be used in fermentation processes or transgenic animals. The aim was to produce recombinant proteins to for instance cow milk.
- The promoter used to drive foreign protein production is a strong eucaryotic or virus promoter which is in their invention controlled by the tetracycline regulatory system.

Peroxidases or oxidases can be used together with compounds such as luminol or acridines (for instance lucigenin) to yield luminescent signals suitable for a detection system described here. Enzymatically generated chemiluminescence offers great sensitivity and rapid detection, too, in assays described in this invention. Thermally stable dioxetanes (such as AMPPD and Lumigen PPD) can be enzymatically (such as alkaline phosphatase or beta-galactosidase) triggered to produce chemiluminescence (Schaap *et al.*, 1989, Clin. Chem., 35, 1863-64). The only difference to the luciferase enzymes would be that these

gives light emission (chemiluminescence) and the luciferases cleave natural substrates to produce light (bioluminescence).

The reporter genes mentioned lead to a very sensitive detection of gene expression especially those belonging to the luciferase group (Pazzagli et al. (1992) Use of bacterial and firefly luciferases as reporter genes in DEAE-dextran-mediated transfection of mammalian cells. Anal. Biochem. 204, 315-323). Other reporter genes such as chloramphenicol acetyl transferase (CAT) or various enzymes utiling chromogenic substrates (leading to less sensitive detection of the order of several magnitudes) such as β-galactosidase, alkaline phosphatase and β-glucuronidase have also been used widely.

A method to study various tetracyclines and their mode of action was developed by Chopra et al. (Chopra aet al., (1990) Sensitive biological detection method for tetracyclines using a *tetA-lacZ* fusion system. Antimicrob. Agents Chemother., 34, 111-116). The assay system developed in this study was based on expression of β-galactosidase gene inserted under the control of tetA-gene. The method resulted in less sensitive detection of tetracyclines compared to the invention described here. However in order to obtain maximum sensitivities Chopra et al. showed that it was necessary to add cyclic AMP (cAMP) to the medium which is an extremely expensive molecule to be used in routine applications. Furthermore, the method described by Chopra et al. contains a cell disruption stage by sonication in order to assay for the reporter gene activity, β-galactosidase, which step is not practical. Instead, the method described in this invention does not contain any cell disruption. The activity of luciferase can be measured directly from living cells in real-time and in the case of pTetLux1 there is no need of addition of any substrates. Therefore, promoter activation due to the

continuously.

EXPERIMENTS

As cloning hosts and in antibiotic residue measurements various *E. coli* MC1061 (*c*I+, *ara*D139, Δ(*ara-leu*)7696, *lac*X74, *gal*U, *gal*K, *hsr*, *hsm*, *str*A) (Casadaban & Cohen, J. Mol. Biol., 138, 1980, 179-207), BW322 (CGSC, *rfa*210::Tn10, *thi*-1, *rel*A1, *spo*T1, *pyr*E) and K-12 (M72 Sm^R *lac*Zm-Δ*biouvr*B, *trp*EA2 (*N*am7*N*am53*c*I857 HI) (Remaut *et al.*, 1981, GENE, 15, 81-93) can be used. Especially the strain LH530 (Hirvas L, et al., 1997, Microbiology, 143, 73-81) which has a decreased rate of lipid A biosynthesis. It has proven to be hypersusceptible to many different antibiotics.

Cells were grown on appropriate minimal agar-plates and were kept maximally one month at +4°C after which new plates were stroken. The strains were kept also in 15% glycerol at -70°C, where from growth was started through minimal plates. The cells were first cultivated in 5 ml of 2xTY medium (16 g Bacto tryptone, 8 g Yeast extract, 8 g NaCl, H_2O ad 1 l, pH 7.4, with appropriate antibiotic) 10 h at 30°C in a shaker after which the cultivation was transferred to a bigger volume for 10 h same medium.

Construction of tetracycline-responsive sensor plasmids:

To construct a recombinant DNA vector carrying luciferase genes under the control of a tetracycline responsive elements two new vectors were created. In the first one modified firefly luciferase gene from vector pBLuc* (Bonin et al., 1994, *Photinus pyralis* luciferase: vectors that contain a modified *luc* coding sequence allowing convenient transfer into other systems. Gene, 141, 75-77.) was excised by using restriction enzymes *Xba*I and *Hin*DIII and the 1.7 kb fragment was isolated from LGT-agarose gel and purified

containing the entire *Photinus pyralis* luciferase gene was ligated using T4-DNA-ligase enzyme to vector pASK75 (Skerra A, "Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in Escherichia coli", Gene 1994 Dec 30;151(1-2):131-135) which was previously restricted with the same restriction enzymes *Xba*I and *Hin*DIII and calf intestinal phosphatase treated to remove the protruding phosphate groups in order to prevent self-ligation. The resulting ligation mixture was incubated 3 hours at room temperature after which one µl of the mixture was electroporated according to Dower *et al.* (Dower, W. J.; Miller, J. F.; Ragsdale, C. W. *Nucl. Acid. Res.* 1988, 16, 6126-6144) into electrocompetent *E. coli* MC1061 cells. A plasmid was extracted from one of the colonies obtained and checked for the estimated structure by appropriate restriction enzyme digestions and agarose gel electrophoretic techniques. The plasmid obtained was named as pTetLuc1.

The plasmid containing the luxCDABE genes of *Photorhabdus luminescens* under the control of tetracycline responsive element was created as follows: Plasmid pASK75 was cut with restriction enzyme *Eco*RI and CIP-treated. The linearized plasmid was separated on a LGT-agarose gel electrophoresis and the agarose was removed by using the Qiagen kit. The lux operon was excised with EcoRI from plasmid pCGLS-11 (Frackman et al., 1990, "Cloning, organization and expression of the bioluminescence genes of *Xenorhabdus luminescens*", J. Bacteriology., 172, 5767-5773), gel purified as above and ligated to pASK75 by using T4-DNA-ligase at 16 °C overnight. The ligation mixture was electroporated into *E. coli* MC1061 cells as described above and correct transformants were screened for their ability to produce light (as measured with a BioOrbit 1250 manual luminometer) which production was increased in the presence of 1 µg/ml of tetracycline-HCl. The plasmid was further

structure was named as pTetLux1. All the DNA-manipulations were performed according to Sambrook *et al.*, "Molecular Cloning: A laboratory Manual, Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

The vector pASK75 was utilized in the construction of tet-sensor plasmids shown in this invention. The vector pASK75 was originally developed for protein production and purification purposes. It contains a signal sequence for secretion of the recombinant protein into the periplasmic space of *E. coli*. Also a C-terminal fusion between a purification tail, the Strept-tag, was incorporated into the vector to facilitate purification of recombinant protein using streptavidin affinity agarose gel chromatography. The element controlling recombinant gene expression in the vector is *tet*A promoter/operator system that allows efficient regulation of the expression, which in Skerra's paper was described for the production and one-step purification of a murine single-chain antibody fragment.

Transfer of the tetracycline sensor vectors to the antibiotic sensitive *E. coli* strain:

Either pTetLux1 or pTetLuc1 was transformed into *E. coli* LH530 cells by electroporation as described above. The transformed cells were restreaked on agar plates and kept maximally for 2 weeks at +4 °C after which a new plate was streaked.

Use of the manipulated *E. coli* in tetracycline determination methods:

Example 1

Freeze-dried *E. coli* K-12/pTetLux1 were reconstituted with 1.0 ml of L-broth and bacteria were diluted 1:10 with 25 mM

MES buffer in M9 minimal medium, pH6,0. 190 µl bacterial suspension was added to microtiter plate wells containing 10 µl of tetracycline dilutions. The plate was incubated 90 minutes at 37°C after which the plate was measured with Labsystems Luminoskan luminometer. As seen from Figure 7 the sensitivity of the assay of tetracycline is very high and comparable to that of fresh cells.

Example 2

Two different types of sensor DNA vector construct were compared. Strains *E. coli* K-12/pTetLux1 and *E. coli* K-12/pTetLuc1 were cultivated in L-broth media until optical density measured at 600nm (OD600) was 1.5. The cells were diluted 1 to 50 with 25 mM MES-buffer in M9 minimal medium, pH 6.0 (Sambrook *et al.*, 1989, Cold Spring Harbor Laboratory, Cold Spring Harbor) and 190 µl was added to a microtitration plate wells and 10 µl of sample dilution of tetracycline was added. After a 60 min incubation at 37 °C the light emission was measured using a Labsystems Luminoskan luminometer. Figure 8 shows the bioluminescence dose-response curve as a function of tetracycline added. As seen from the figure both systems (bacterial or insect luciferase give roughly equal sensitivity of tetracycline detection.

One is able to use different luciferases instead of bacterial luciferase from *P. luminescens* without losing sensitivity or other performance of the test. There is shown in Figure 8 an analogous measurement as shown in Figure 4b. In the plasmid used in this test (pTetLuc1) the bacterial luciferase was compensated with firefly luciferase as described in Figure 3a. The test was done essentially as with bacterial luciferase except that after the cells had been incubated with or without tetracycline 10 minutes at 37°C the cells were measured for light production after 15 minutes incubation time at 37°C by

adding 100 ul of solution containing 1 mM D-luciferin, in 0.1 M Na-citrate buffer, pH 5.0. Thereafter the light production was measured using a manual luminometer 1250 (LKB-Wallac, Turku, Finland). As can be seen from the Figure 8 the sensitivity of the method to detect tetracycline hydrochloride is extremely high and comparable to the detection made with bacterial luciferase.

Example 3

A lipemic pig serum was spiked at different concentrations of tetracycline, chlorotetracycline and oxytetracycline. Fresh *E. coli* K-12/pTetLux1 were dliluted 1:50 with 25 mM MES buffer in M9 minimal medium, pH 6,0. 100 µl bacterial suspension was added to microtiter plate wells containing 100 ul of pig serum spiked with different tetracyclines. The plate was incubated 90 minutes at 37 °C after which the plate was measured with Labsystems Luminoskan luminometer. As seen from Figure 9 the sensitivity of the assay of different tetracyclines in pig serum matrix is very high.

Example 4

Tetracyclines will form chelate complexes with Ca^{2+} and Mg^{2+} in samples (e.g. milk), and loose their antimicrobial and induction activity in our assay system. Tetracyclines can be displaced from cation chelates by using strong chelating agents such as EDTA. There is shown in the Figure 10 the assay of tetracycline from a milk sample, which is spiked at different concentrations of tetracycline. Different amounts of EDTA were added to milk samples and this kind of displacement of cation-tetracycline complex clearly improved the sensitivity of the assay. In the assay we used freeze-dried *E. coli* K12/pTetLux1 that were reconstituted with L-broth 10 minutes in room temperature before the assay.

Example 5

In Figure 11 is shown the kinetics of bacterial bioluminescence after exposure of *E. coli* K-12/pTetLux1 to different dilutions of tetracycline antibiotics. The specific induction of tetracycline is very fast and specific light emission is seen already in 10 minutes measuring point in the assay.

It will be appreciated that the methods of the present invention can be incorporated in the form of a variety of embodiments, only a few of which are disclosed herein. It will be apparent for the specialist in the field that other embodiments exist and do not depart from the spirit of the invention. Thus, the described embodiments are illustrative and should not be construed as restrictive.

CLAIMS

1. A method for the detemination of a tetracycline in a sample characterized in that
- the sample is brought into contact with cells encompassing a DNA vector including a nucleotide sequence encoding a light producing enzyme under transcriptional control of tetracycline repressor and tetracycline promoter,
- detecting the luminescense emitted from the cells, and
- comparing the emitted luminescence to the luminescence emitted from cells in a control containing no tetracycline.

2. The method according to claim 1 characterized in that the cells are prokaryotic cells, preferably *Escherichia coli*.

3. The method according to claim 1 or 2 characterized in that the DNA vector is a plasmid containing the luxCDABE ~~genes, tetracycline repressor (TetR) and tetracycline~~ promotor (TetA) from *Tn*10.

4. The method according to claim 3 characterized in that the DNA vector is the plasmid pTetLux1.

5. The method according to claim 1 or 2 characterized in that
~~- the DNA vector is a plasmid containing the insect~~ luciferase gene, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10, and that
- D-luciferin is added to the mixture of the sample and the cells in order to initiate the luminescence of the cells.

6. The method according to claim 5 characterized in that the DNA vector is the plasmid pTetLuc1.

7. The method according to any of the claims 1 - 6 characterized in that the sensitivity of the analysis with respect to the tetracycline is controlled by
- increasing or decreasing the concentration of divalent metal ions, e.g. magnesium ions, or
- adjusting the pH, or
- combinated adjusting of the divalent metal ion concentration and the pH.

8. The method according to any of the claims 1 - 6 characterized in that the sensitivity of the analysis with respect to the tetracycline derivative is increased by the use of cells which are especially antibiotic sensitive mutant strains.

9. The method according to any of the claims 1 - 8 characterized in that the luminescence is measured using an X-ray or polaroid film, a CCD-camera, a liquid scintillation counter or a luminometer.

10. The method according to any of the claims 1 - 9 characterized in that the sample to be analysed is milk, fish, meat, infant formula, eggs, honey, vegetables, serum, plasma, whole blood or the like.

11. A recombinant prokaryotic cell characterized in that it encompasses a DNA vector including a nucleotide sequence encoding a light producing enzyme, tetracycline repressor and tetracycline promoter.

12. The cell according to claim 11 characterized in that the DNA vector is a plasmid containing either
- the luxCDABE genes, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10 , or
- the insect luciferase gene, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10.

that it is *Escherichia coli*.

14. The cell according to claim 12, 13 or 14, characterized in that it is in dried form, e.g. in lyophilized form.

15. A plasmid characterized in that it comprises either
- the luxCDABE genes, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10 , or
- the insect luciferase gene, tetracycline repressor (TetR) and tetracycline promotor (TetA) from *Tn*10.

16. A plasmid according to claim 15 characterized in that it is pTetLux1 or pTetLuc1.

(57) ABSTRACT

The invention relates to a method for the detemination of a tetracycline in a sample. The method is characterized in that - the sample is brought into contact with cells encompassing a DNA vector including a nucleotide sequence encoding a light producing enzyme under transcriptional control of tetracycline repressor and tetracycline promoter,
- detecting the luminescense emitted from the cells, and
- comparing the emitted luminescence to the luminescence emitted from cells in a control containing no tetracycline.

The invention also concerns recombinant prokaryotic cells capable of emitting light in response to the existence of a tetracycline in a sample. Furthermore, the invention relates to novel DNA vectors useful for the construction of said prokaryotic cells.

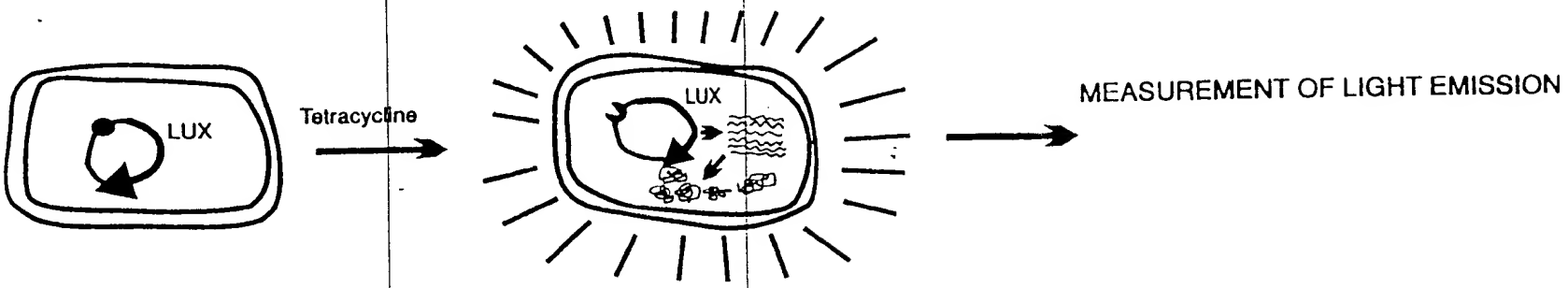

LUX
Tetracycline
LUX
MEASUREMENT OF LIGHT EMISSION

FIG. 1a

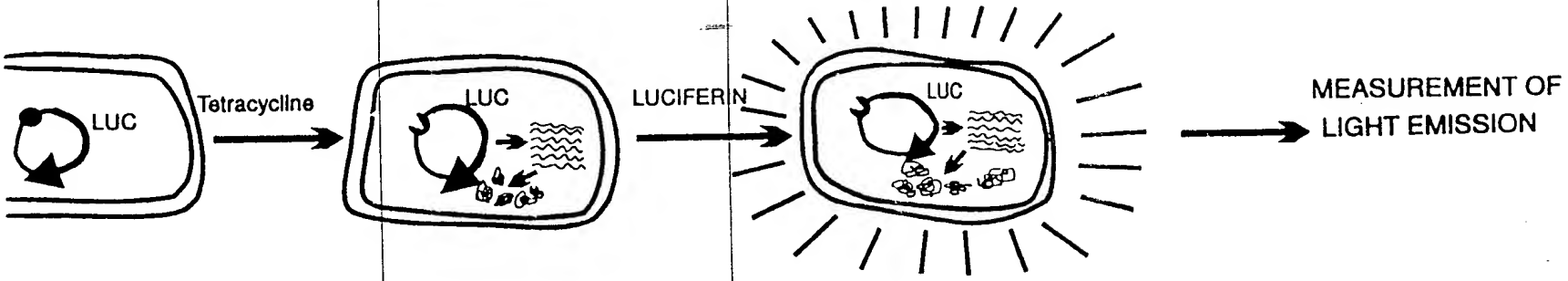
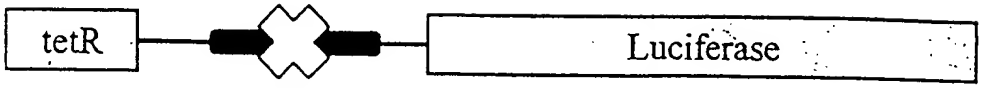

LUC
Tetracycline
LUC
LUCIFERIN
LUC
MEASUREMENT OF
LIGHT EMISSION

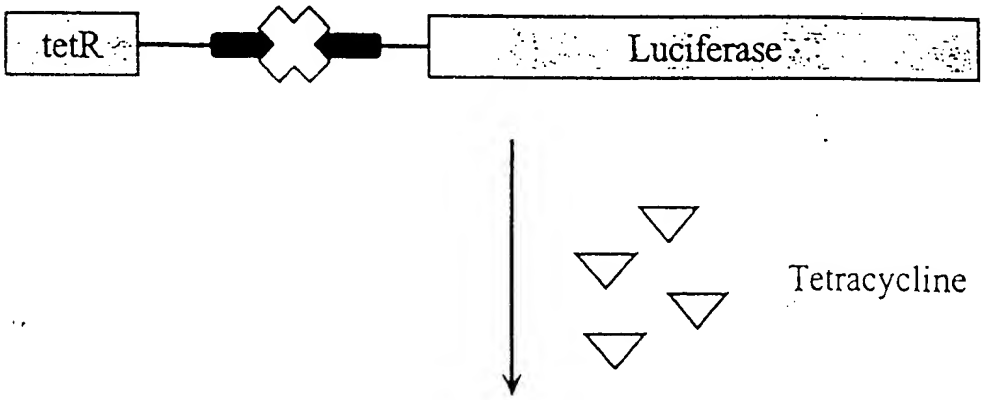
FIG. 1b

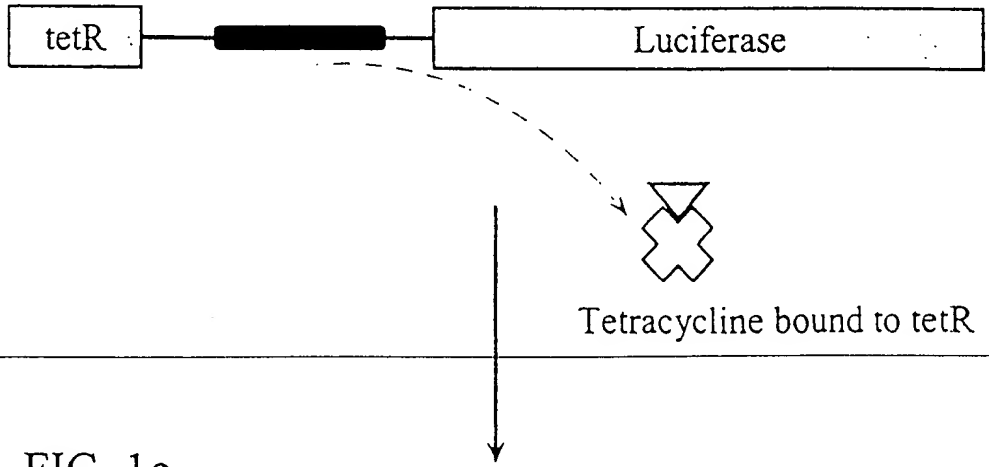
A. No Protein Expression



tetR
Bound to tetA

B. Protein Expression





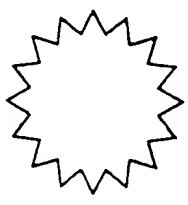
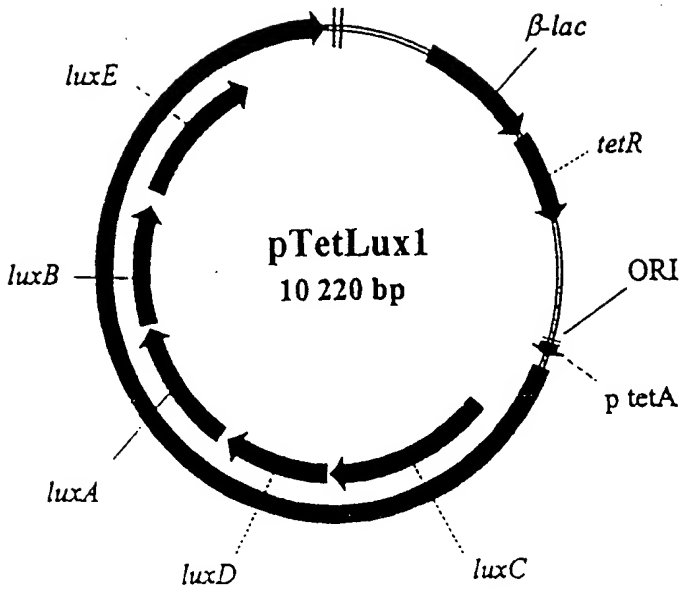


FIG. 1c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tetR
pTetLux1
10 220 bp
luxB
ORI
p tetA
luxA
luxD
luxC

FIG. 2a

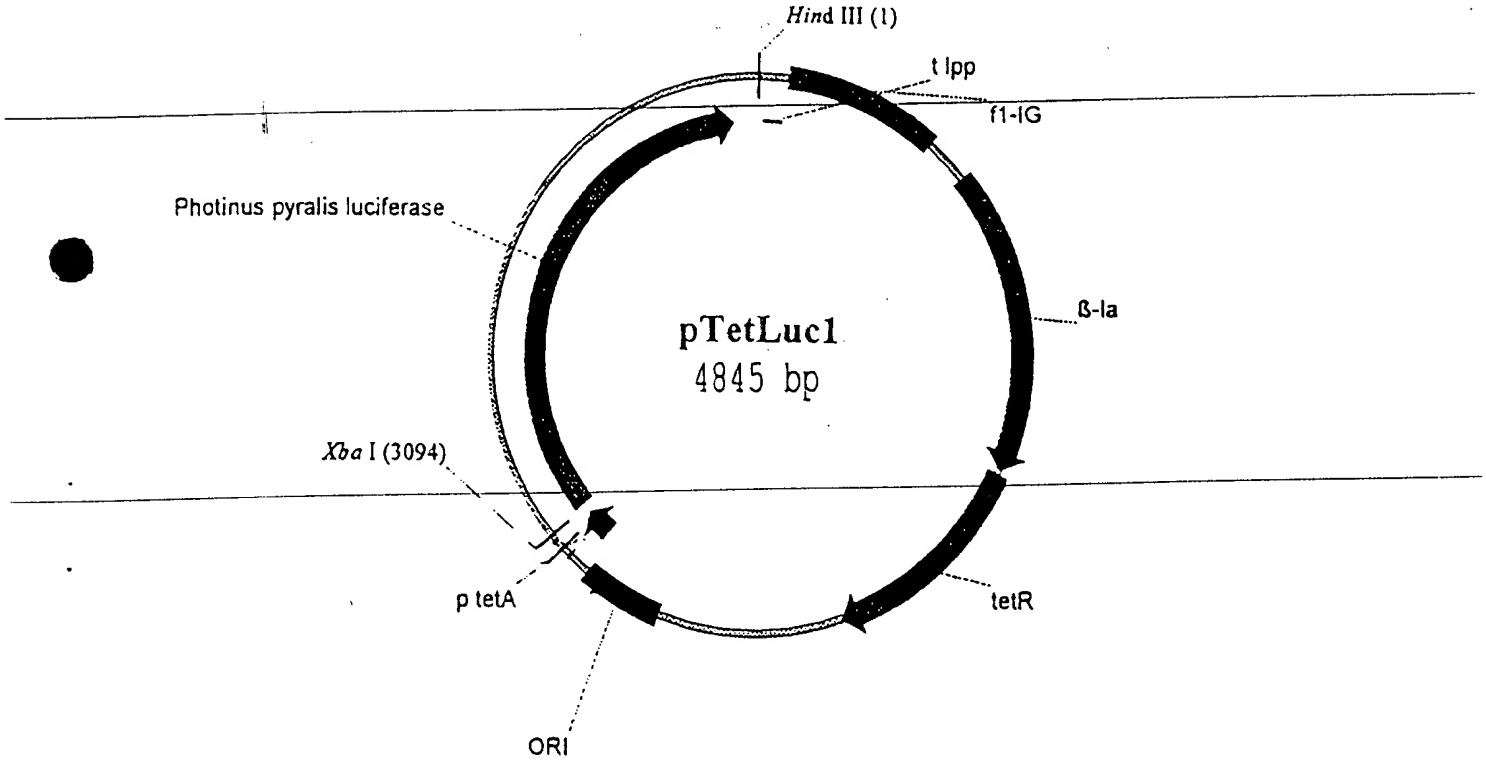
Hind III (1)
t lpp
f1-IG
Photinus pyralis luciferase
ß-la
pTetLuc1
4845 bp
Xba I (3094)
p tetA
tetR
ORI

FIG. 3a

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5251  GGGAAACGCT GCCAGAAGAA AACAGCCCAA AGAGAAAGAA TGCCATTATT
5301  ATTGCGTCTG GTTTTGCCCG CAGGATGGAT CATTTTGCTG GTCTGGCGGA
5351  ATATTTATCG CGGAATGGAT TTCATGTGAT CCGCTATGAT TCGCTTCACC
5401  ACGTTGGATT GAGTTCAGGG ACAATTGATG AATTTACAAT GTCTATAGGA
5451  AAGCAGAGCT TGTTAGCAGT GGTTGATTGG TTAACTACAC GAAAAATAAA
5501  TAACTTCGGT ATGTTGGCTT CAAGCTTATC TGCGCGGATA GCTTATGCAA
5551  GCCTATCTGA AATCAATGCT TCGTTTTTAA TCACCGCAGT CGGTGTTGTT
5601  AACTTAAGAT ATTCTCTTGA AAGAGCTTTA GGGTTTGATT ATCTCAGTCT
5651  ACCCATTAAT GAATTGCCGG ATAATCTAGA TTTTGAAGGC CATAAATTGG
5701  GTGCTGAAGT CTTTGCGAGA GATTGTCTTG ATTTTGGTTG GGAAGATTTA
5751  GCTTCTACAA TTAATAACAT GATGTATCTT GATATACCGT TTATTGCTTT
5801  TACTGCAAAT AACGATAATT GGGTCAAGCA AGATGAAGTT ATCACATTGT
5851  TATCAAATAT TCGTAGTAAT CGATGCAAGA TATATTCTTT GTTAGGAAGT
5901  TCGCATGACT TGAGTGAAAA TTTAGTGGTC CTGCGCAATT TTTATCAATC
5951  GGTTACGAAA GCCGCTATCG CGATGGATAA TGATCATCTG GATATTGATG
6001  TTGATATTAC TGAACCGTCA TTTGAACATT TAACTATTGC GACAGTCAAT
6051  GAACGCCGAA TGAGAATTGA GATTGAAAAT CAAGCAATTT CTCTGTCTTA
6101  AAATCTATTG AGATATTCTA TCACTCAAAT AGCAATATAA GGACTCTCTA
6151  TGAAATTTGG AAACTTTTTG CTTACATACC AACCTCCCCA ATTTTCTCAA
6201  ACAGAGGTAA TGAAACGTTT GGTTAAATTA GGTCGCATCT CTGAGGAGTG
6251  TGGTTTTGAT ACCGTATGGT TACTGGAGCA TCATTTCACG GAGTTTGGTT
6301  TGCTTGGTAA CCCTTATGTC GCTGCTGCAT ATTTACTTGG CGCGACTAAA
6351  AAATTGAATG TAGGAACTGC CGCTATTGTT CTTCCCACAG CCCATCCAGT
6401  ACGCCAACTT GAAGATGTGA ATTTATTGGA TCAAATGTCA AAAGGACGAT
6451  TTCGGTTTGG TATTTGCCGA GGGCTTTACA ACAAGGACTT TCGCGTATTC
6501  GGCACAGATA TGAATAACAG TCGCGCCTTA GCGGAATGCT GGTACGGGCT
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6551  GATAAAGAAT GGCATGACAG AGGGATATAT GGAAGCTGAT AATGAACATA
6601  TCAAGTTCCA TAAGGTAAAA GTAAACCCCG CGGCGTATAG CAGAGGTGGC
6651  GCACCGGTTT ATGTGGTGGC TGAATCAGCT TCGACGACTG AGTGGGCTGC
6701  TCAATTTGGC CTACCGATGA TATTAAGTTG GATTATAAAT ACTAACGAAA
6751  AGAAAGCACA ACTTGAGCTT TATAATGAAG TGGCTCAAGA ATATGGGCAC
6801  GATATTCATA ATATCGACCA TTGCTTATCA TATATAACAT CTGTAGATCA
6851  TGACTCAATT AAAGCGAAAG AGATTTGCCG GAAATTTCTG GGGCATTGGT
6901  ATGATTCTTA TGTGAATGCT ACGACTATTT TTGATGATTC AGACCAAACA
6951  AGAGGTTATG ATTTCAATAA AGGGCAGTGG CGTGACTTTG TATTAAAAGG
7001  ACATAAAGAT ACTAATCGCC GTATTGATTA CAGTTACGAA ATCAATCCCG
7051  TGGGAACGCC GCAGGAATGT ATTGACATAA TTCAAAAAGA CATTGATGCT
7101  ACAGGAATAT CAAATATTTG TTGTGGATTT GAAGCTAATG GAACAGTAGA
7151  CGAAATTATT GCTTCCATGA AGCTCTTCCA GTCTGATGTC ATGCCATTTC
7201  TTAAAGAAAA ACAACGTTCG CTATTATATT AGCTAAGGAG AAAGAAATGA
7251  AATTTGGATT GTTCTTCCTT AACTTCATCA ATTCAACAAC TGTTCAAGAA
7301  CAAAGTATAG TTCGCATGCA GGAAATAACG GAGTATGTTG ATAAGTTGAA
7351  TTTTGAACAG ATTTTAGTGT ATGAAAATCA TTTTTCAGAT AATGGTGTTG
7401  TCGGCGCTCC TCTGACTGTT TCTGGTTTTC TGCTCGGTTT AACAGAGAAA
7451  ATTAAAATTG GTTCATTAAA TCACATCATT ACAACTCATC ATCCTGTCGC
7501  CATAGCGGAG GAAGCTTGCT TATTGGATCA GTTAAGTGAA GGGAGATTTA
7551  TTTTAGGGTT TAGTGATTGC GAAAAAAAAG ATGAAATGCA TTTTTTTAAT
7601  CGCCCGGTTG AATATCAACA GCAACTATTT GAAGAGTGTT ATGAAATCAT
7651  TAACGATGCT TTAACAACAG GCTATTGTAA TCCAGATAAC GATTTTTATA
7701  GCTTCCCTAA AATATCTGTA AATCCCCATG CTTATACGCC AGGCGGACCT
7751  CGGAAATATG TAACAGCAAC CAGTCATCAT ATTGTTGAGT GGGCGGCCAA
7801  AAAAGGTATT CCTCTCATCT TTAAGTGGGA TGATTCTAAT GATGTTAGAT
7851  ATGAATATGC TGAAAGATAT AAAGCCGTTG CGGATAAATA TGACGTTGAC
7901  CTATCAGAGA TAGACCATCA GTTAATGATA TTAGTTAACT ATAACGAAGA
7951  TAGTAATAAA GCTAAACAAG AGACGCGTGC ATTTATTAGT GATTATGTTC
8001  TTGAAATGCA CCCTAATGAA AATTTCGAAA ATAAACTTGA AGAAATAATT
8051  GCAGAAAACG CTGTCGGAAA TTATACGGAG TGTATAACTG CGGCTAAGTT
8101  GGCAATTGAA AAGTGTGGTG CGAAAAGTGT ATTGCTGTCC TTTGAACCAA
8151  TGAATGATTT GATGAGCCAA AAAAATGTAA TCAATATTGT TGATGATAAT
8201  ATTAAGAAGT ACCACATGGA ATATACCTAA TAGATTTCGA GTTGCAGCGA
8251  GGCGGCAAGT GAACGAATCC CCAGGAGCAT AGATAACTAT GTGACTGGGG
8301  TGAGTGAAAG CAGCCAACAA AGCAGCAGCT TGAAAGATGA AGGGTATAAA
8351  AGAGTATGAC AGCAGTGCTG CCATACTTTC TAATATTATC TTGAGGAGTA
8401  AAACAGGTAT GACTTCATAT GTTGATAAAC AAGAAATTAC AGCAAGCTCA
8451  GAAATTGATG ATTTGATTTT TTCGAGCGAT CCATTAGTGT GGTCTTACGA
8501  CGAGCAGGAA AAAATCAGAA AGAAACTTGT GCTTGATGCA TTTCGTAATC
8551  ATTATAAACA TTGTCGAGAA TATCGTCACT ACTGTCAGGC ACACAAAGTA
8601  GATGACAATA TTACGGAAAT TGATGACATA CCTGTATTCC CAACATCGGT
8651  TTTTAAGTTT ACTCGCTTAT TAACTTCTCA GGAAAACGAG ATTGAAAGTT
8701  GGTTTACCAG TAGCGGCACG AATGGTTTAA AAAGTCAGGT GGCGCGTGAC
8751  AGATTAAGTA TTGAGAGACT CTTAGGCTCT GTGAGTTATG GCATGAAATA
8801  TGTTGGTAGT TGGTTTGATC ATCAAATAGA ATTAGTCAAT TTGGGACCAG
8851  ATAGATTTAA TGCTCATAAT ATTTGGTTTA AATATGTTAT GAGTTTGGTG
8901  GAATTGTTAT ATCCTACGAC ATTTACCGTA ACAGAAGAAC GAATAGATTT
8951  TGTTAAAACA TTGAATAGTC TTGAACGAAT AAAAAATCAA GGGAAAGATC
9001  TTTGTCTTAT TGGTTCGCCA TACTTTATTT ATTTACTCTG CCATTATATG
9051  AAAGATAAAA AAATCTCATT TTCTGGAGAT AAAAGCGTTT ATATCATAAC
9101  CGGAGGCGGC TGGAAAAGTT ACGAAAAAGA ATCTCTGAAA CGTGATGATT
9151  TCAATCATCT TTTATTTGAT ACTTTCAATC TCAGTGATAT TAGTCAGATC
9201  CGAGATATAT TTAATCAAGT TGAACTCAAC ACTTGTTTCT TTGAGGATGA
9251  AATGCAGCGT AAACATGTTC CGCCGTGGGT ATATGCGCGA GCGCTTGATC
9301  CTGAAACGTT GAAACCTGTA CCTGATGGAA CGCCGGGGTT GATGAGTTAT
9351  ATGGATGCGT CAGCAACCAG TTATCCAGCA TTTATTGTTA CCGATGATGT
9401  CGGGATAATT AGCAGAGAAT ATGGTAAGTA TCCCGGCGTG CTCGTTGAAA
9451  TTTTACGTCG CGTCAATACG AGGACGCAGA AAGGGTGTGC TTTAAGCTTA
9501  ACCGAAGCGT TTGATAGTTG ATATCCTTTG CCTAATTGTA AGTGGAATGC
9551  TTGCGTTATA TAAATCTGAA TGACATCTAC ACTTTACAAA ATTCTCCAAA
9601  ACATCCACAT TTGGGTACTT GATAGAGGTT TATGGGGTTG GCTTAACATT
9651  GTTCTCATTG TTATTATTGG CTCAAAGCAA AAGGAGATAA CATGAAAAAA
9701  TTGGCAGTTA TGCTTGCATT GGGAATGATT AGCTTTGGTG CAATGGCAGT
9751  TGATGGGTAT AAAGATGCAA AGTTTGGCAT GACAGAAGAA GAGTTTCTTT
9801  CGAAGAGGTT ATGTGATTTT GAAAAATTTG AGGGAGATTC TCGAATAGAA
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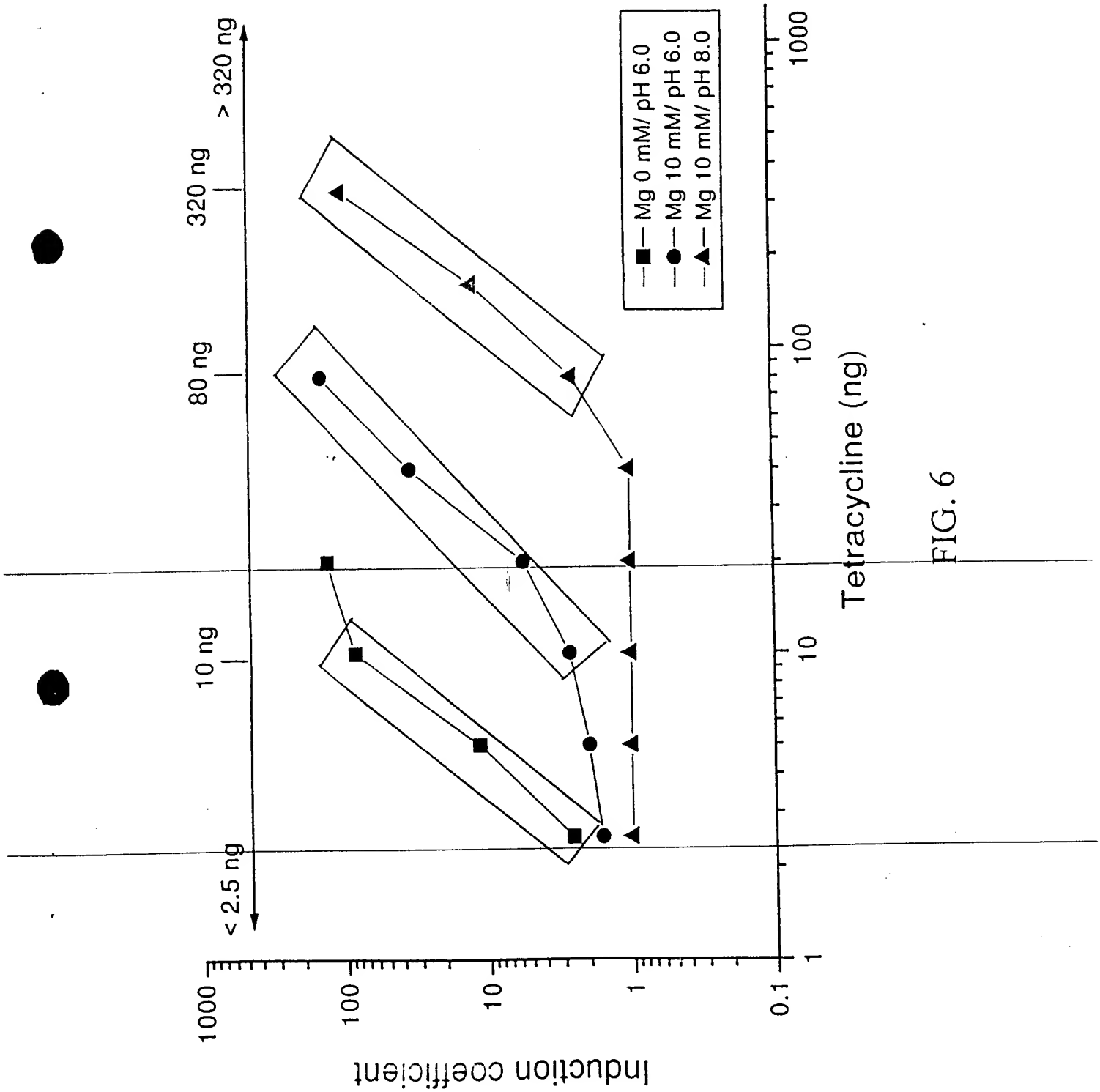
Induction coefficient
1000
100
10
1
0.1
< 2.5 ng
10 ng
80 ng
320 ng
> 320 ng
Mg 0 mM/ pH 6.0
Mg 10 mM/ pH 6.0
Mg 10 mM/ pH 8.0
1
10
100
1000
Tetracycline (ng)

FIG. 6

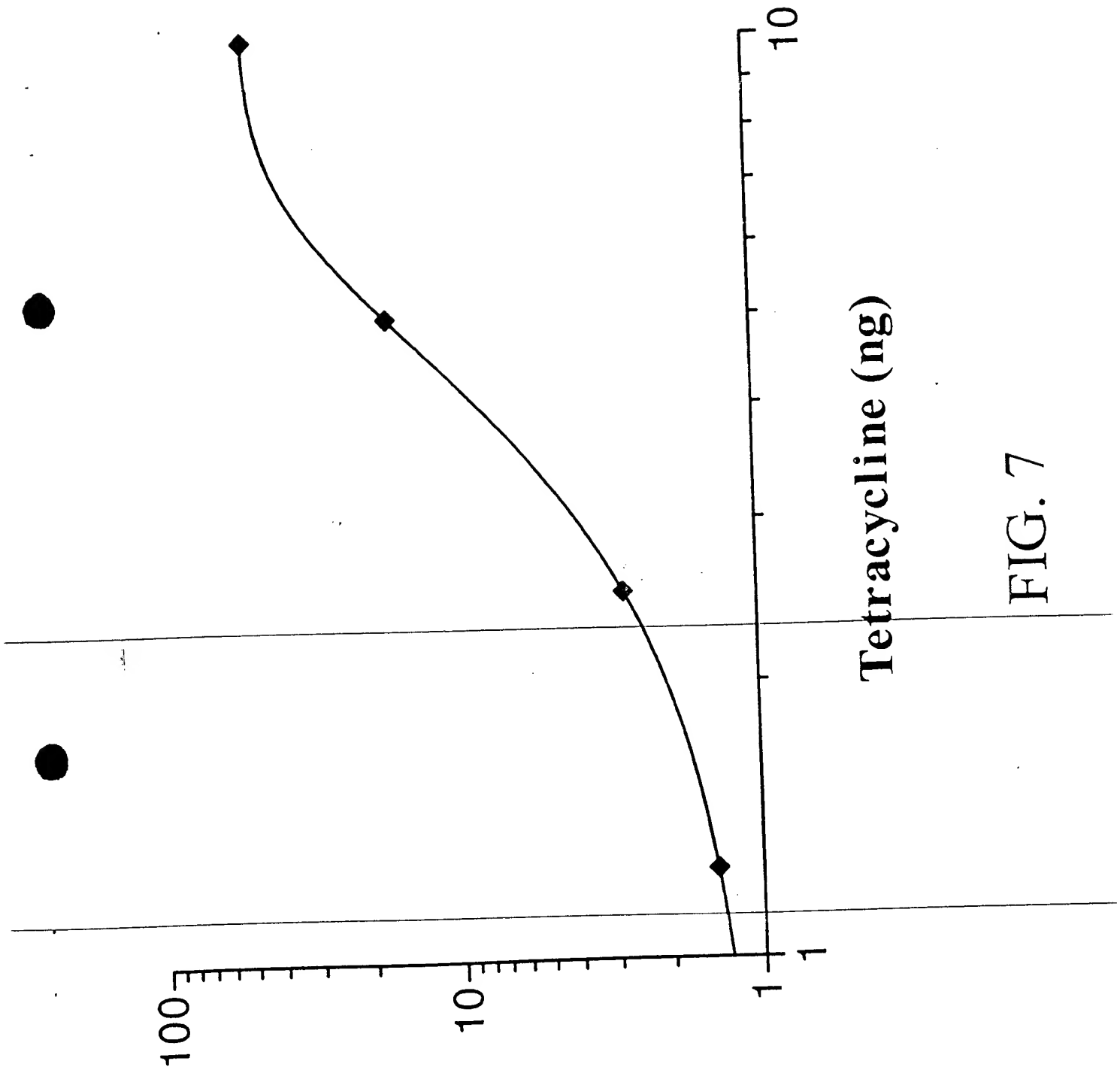

FIG. 7

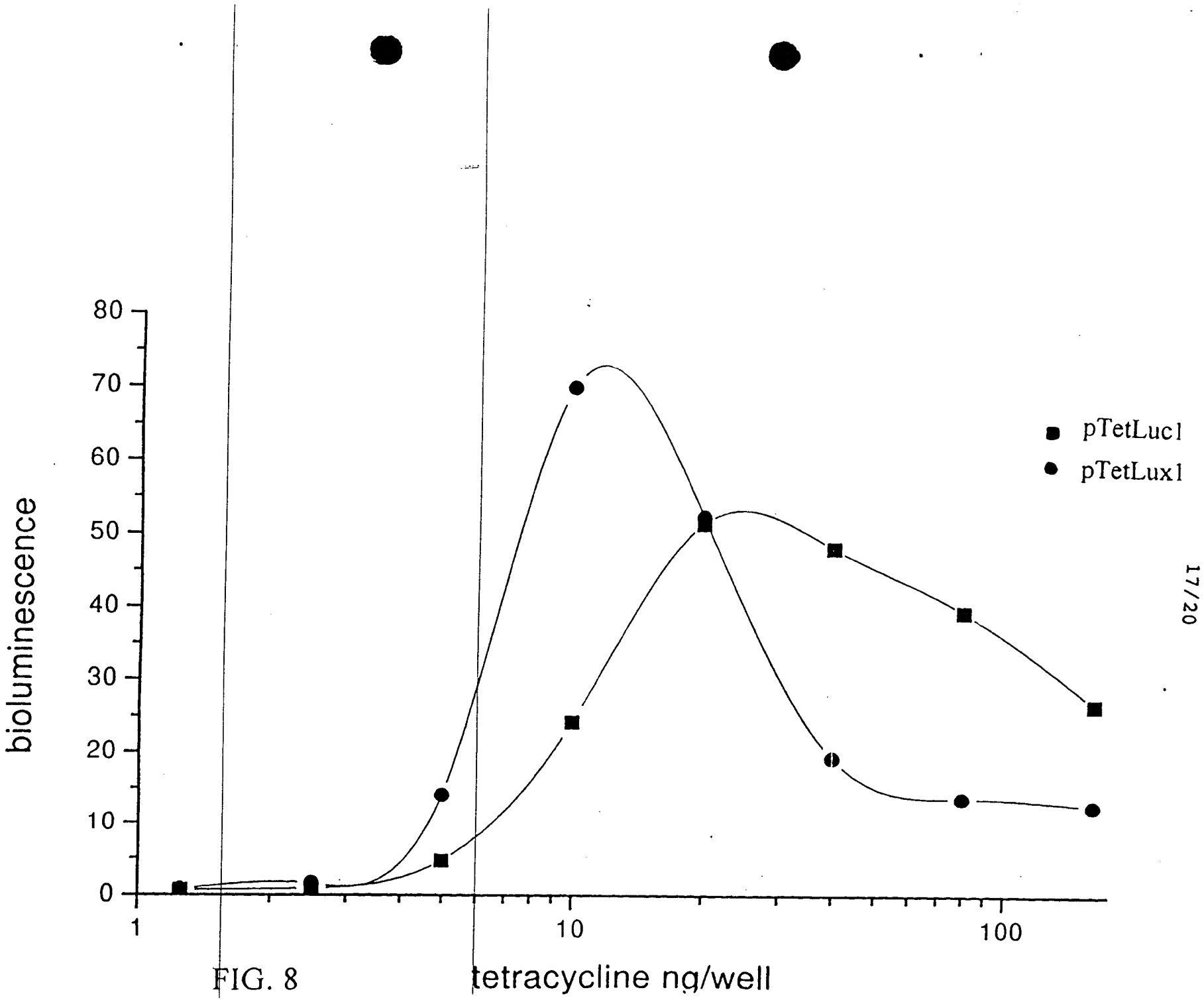
bioluminescence
80
70
60
50
40
30
20
10
0
1
10
100
tetracycline ng/well
pTetLuc1
pTetLux1

FIG. 8

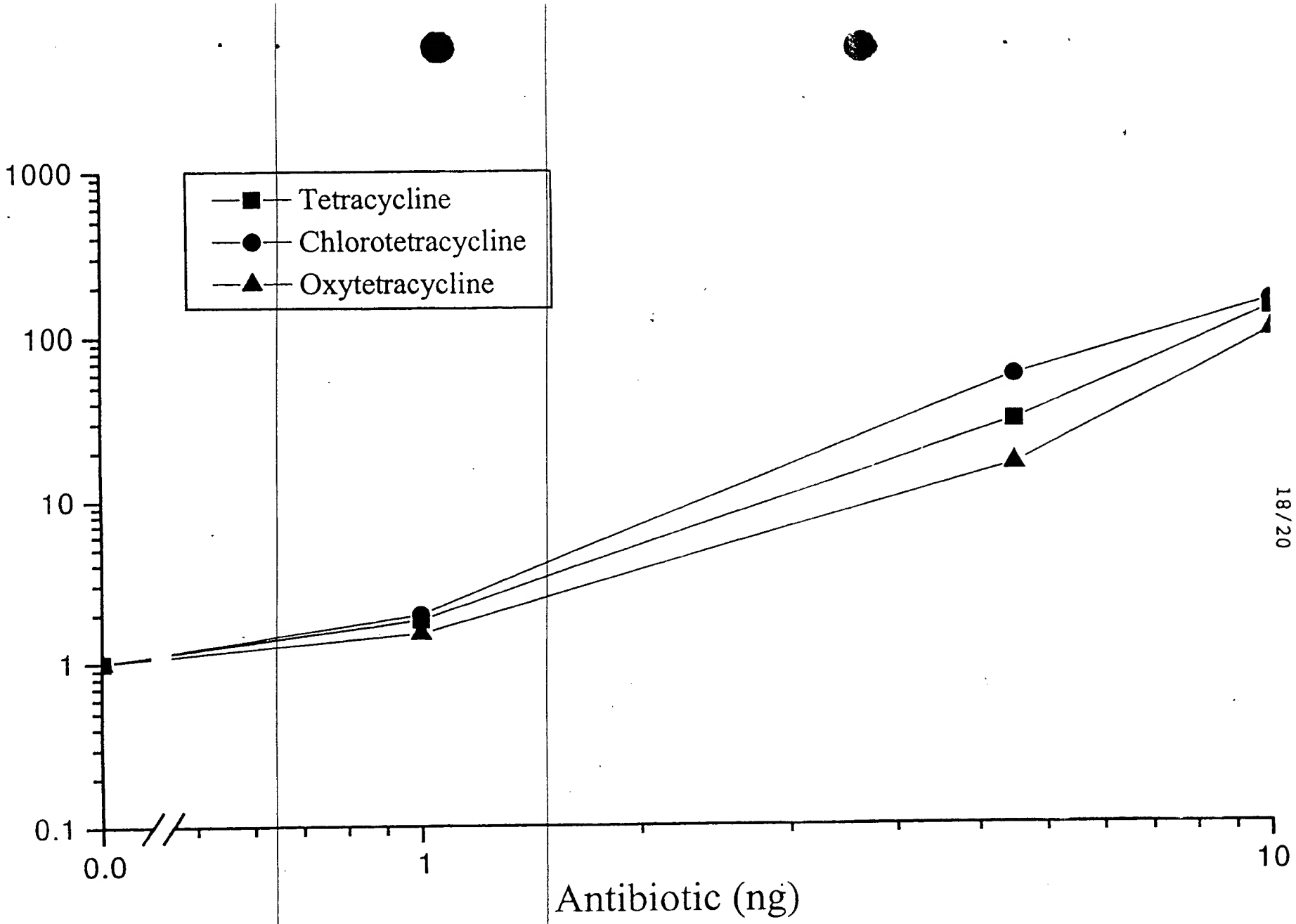
Tetracycline
Chlorotetracycline
Oxytetracycline
1000
100
10
1
0.1
0.0
1
10
Antibiotic (ng)

FIG. 9

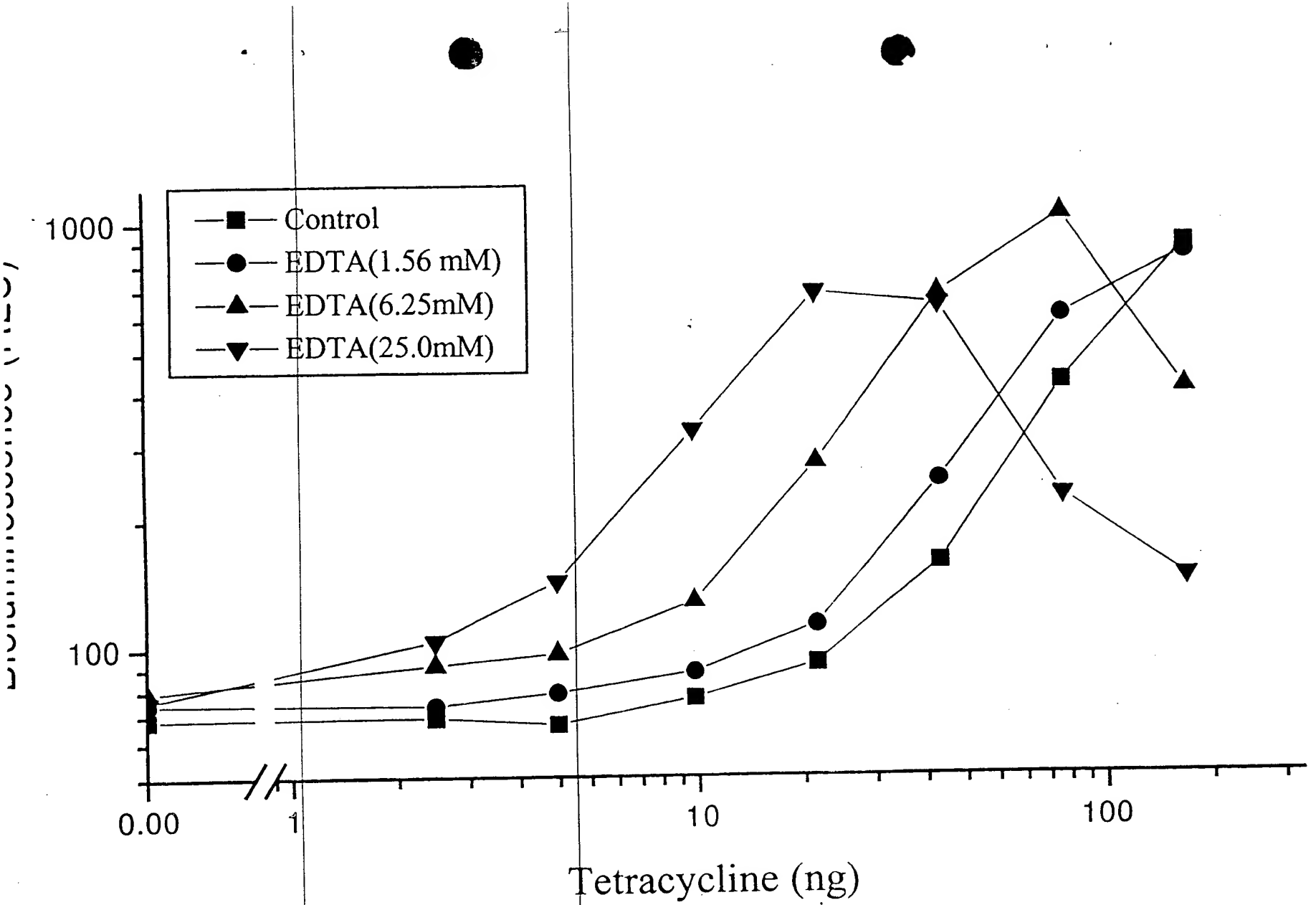
Control
EDTA(1.56 mM)
EDTA(6.25mM)
EDTA(25.0mM)
1000
100
0.00
1
10
100
Tetracycline (ng)

FIG.10

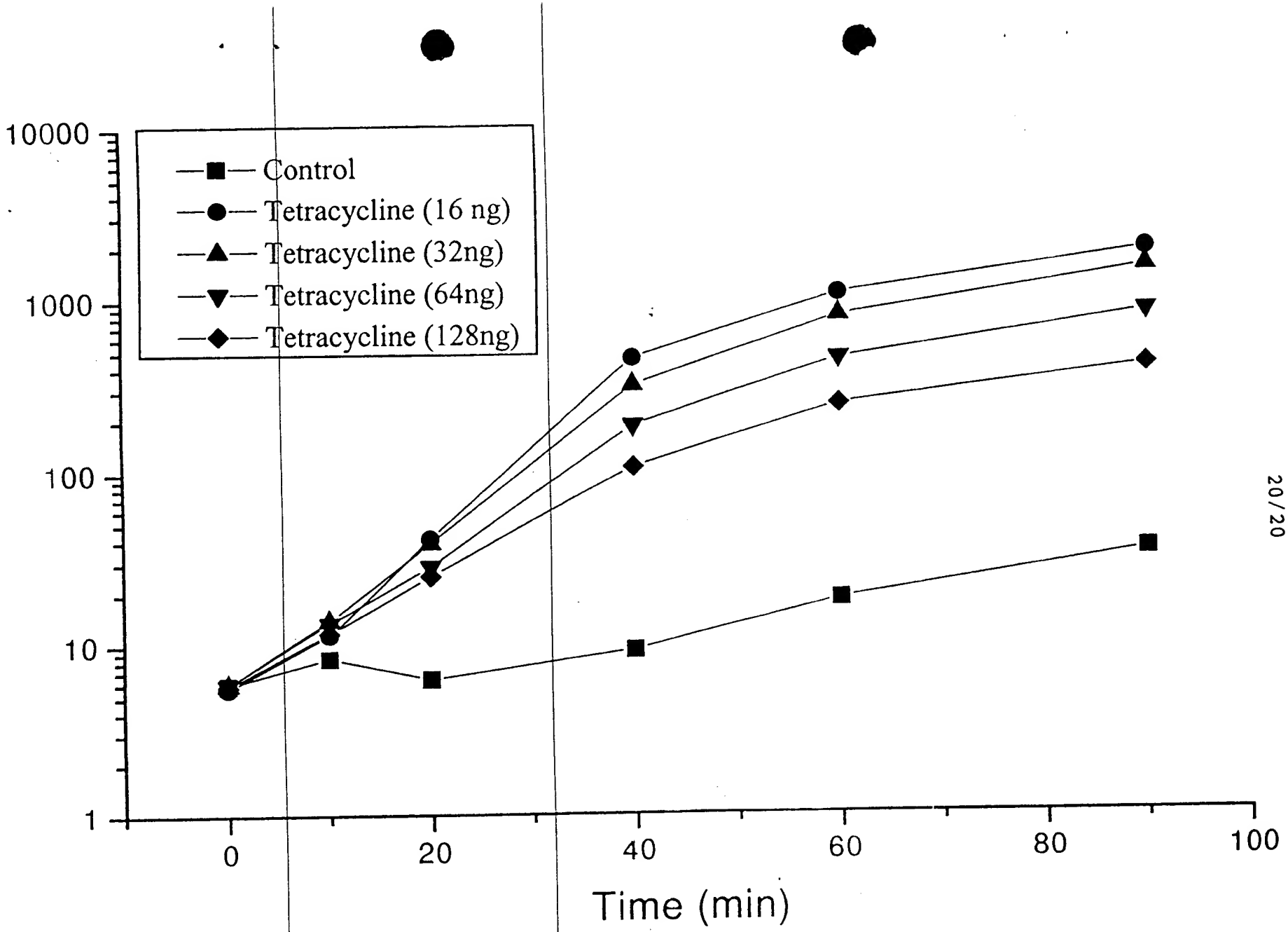
10000
1000
100
10
1
Control
Tetracycline (16 ng)
Tetracycline (32ng)
Tetracycline (64ng)
Tetracycline (128ng)
0
20
40
60
80
100
Time (min)

FIG.11

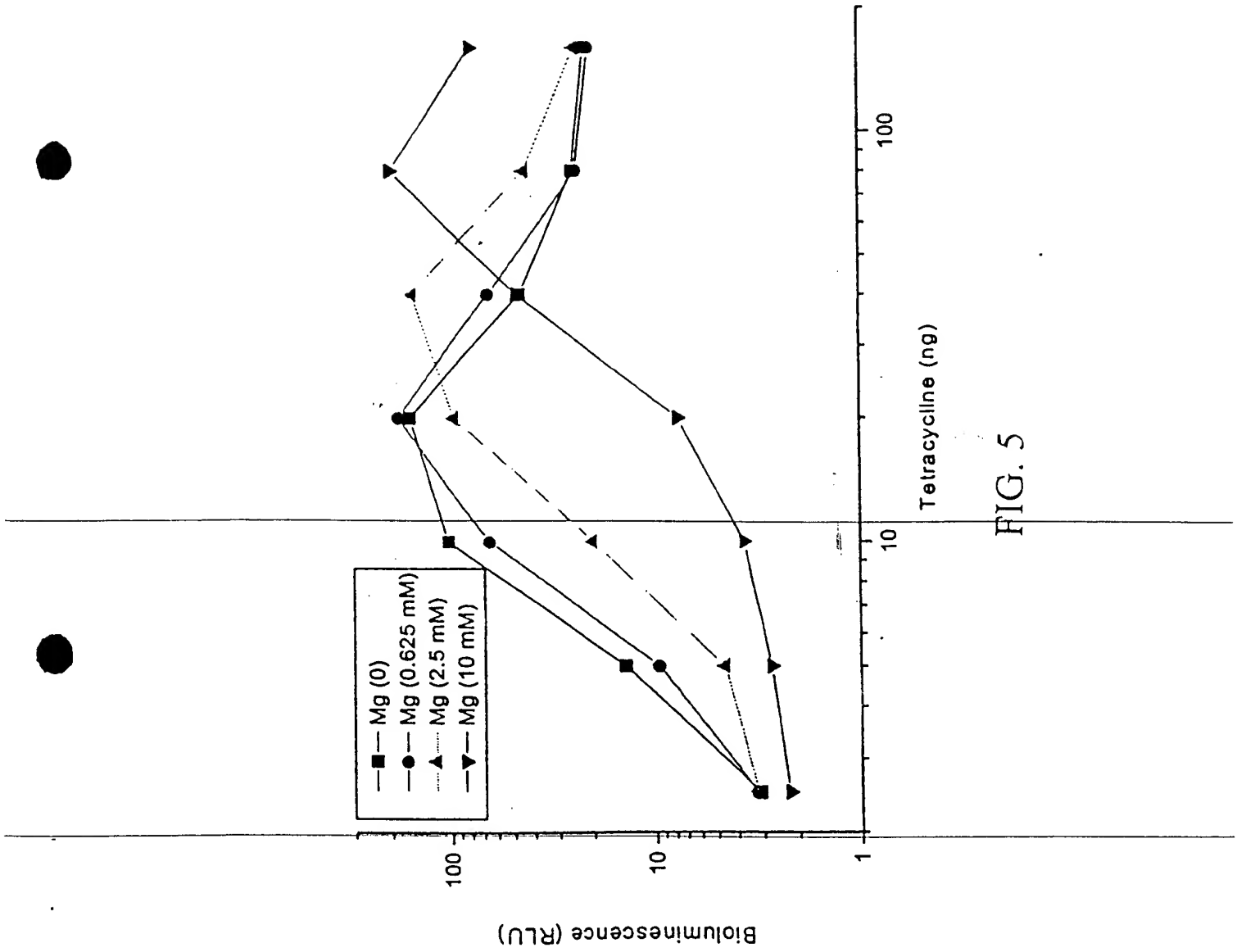
Bioluminescence (RLU)
100
10
1
Tetracycline (ng)
10
100
Mg (0)
Mg (0.625 mM)
Mg (2.5 mM)
Mg (10 mM)

FIG. 5